Brazilian Journal of Microbiology (2012): 356-362
ISSN 1517-8382

CHARACTERIZATION AND LYTIC ACTIVITY OF *PSEUDOMONAS FLUORESCENS* PHAGES FROM SEWAGE

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Submitted: December 04, 2010; Returned to authors for corrections: May 12, 2011; Approved: August 30, 2011.

ABSTRACT

Pseudomonas fluorescens phages from sewage were tested against *P. fluorescens* isolates of soil and sewage. The phages were characterized as to host range, morphology, structural proteins and genome fingerprint. Of the seven phages isolated, one was found to be abundant in sewage (5.9×10^7 pfu/mL), having broad host range, and distinct protein and DNA profile when compared to the other six phages. DNA restriction and protein profiles of the phages and their morphology indicate the diversity in the sewage environment. None of the isolates from the rhizosphere regions of various cultivated soils were susceptible to phages isolated from sewage.

Key words: *Pseudomonas fluorescens*, phages, RFLP, AFM, Protein analysis.

INTRODUCTION

Pseudomonas fluorescens is a diverse community of bacteria, found in wide range of habitats including soil, water and in industrial environments (34). Bacteriophages, the most abundant life forms on earth play major roles in bacterial ecology, adaptation to novel environments, and in bacterial evolution and pathogenesis (23, 10). They have shown good sensitivity and specificity in detecting bacterial pathogens (15). In soil environment *P. fluorescens* is known to improve plant growth and health. (8,17,24). *P. fluorescens* phages are thought to be ecologically important in controlling bacterial number and activity, affecting composition and diversity of beneficial bacterial populations thereby affecting agricultural productivity. Earlier studies have reported isolation of fluorescent *Pseudomonas* phages from different environments (1,5,9). According to the first ever global survey of wastewater irrigation untreated sewage is used to irrigate 10 percent of the world's crops. Sewage should be treated to remove pathogens to make it safe for irrigation (33). This study reports the diversity of *P. fluorescens* phages in sewage with reference to genome size, host range, structural proteins and morphology and their lytic activity on soil bacteria.

MATERIALS AND METHODS

Sample Collection

Sewage sample was obtained from the inlet of the sewage treatment plant located in Sulur, Coimbatore, India. The samples were immediately transported to the laboratory in

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collection bottles within 2 hrs and kept at 4°C prior to processing. The samples were clarified by filtration through cheese cloth and used for further analysis. Soil samples were collected at the depth of 2m from the cultivated lands nearby the sewage treatment plant and stored at 4°C.

Isolation of bacteria from soil and sewage

One mL of sewage sample was added to 9 mL sterile water which makes 10^{-1} dilution and subsequently diluted up to 10^{-6}. Serial dilutions of soil samples were also prepared by adding one gram of soil into 9 mL of sterile water (10^{-1} dilution) and dilutions were made up to 10^{-6}. The diluted samples were plated on King's B agar medium and incubated at 30°C for 48 hrs. Distinct colonies showing fluorescence under UV light at 360 nm were picked and streaked on KB agar medium to check the purity.

P. fluorescens isolated from sewage and from each of the twelve soil samples were subjected to biochemical tests as reported earlier Goszozynska et al (12) and Pickett et al (28). *P. fluorescens* MTCC 103 and *P.aeruginosa* MTCC 2474 were used as a referral strains. The bacterial cultures were grown at 30°C and 42°C with King's B agar medium.

Bacteriophage isolation

P. fluorescens phages isolated from sewage with sewage bacterial isolate as host was enriched by the modified method of Smith and Huggins (35). Briefly, 50 mL of LB broth and 50 mL of tryptone broth were added to 150 ml of the sewage water and incubated for 1 hr at 30°C. To this 5 ml of 24 hr *P. fluorescens* broth culture was added and grown for 16 hours at 30°C. After centrifugation the supernatant was filtered through 0.22 µm membrane filter and stored at 4°C. 100µl of diluted lysate mixed with 100µl of overnight bacterial culture were mixed with 0.7% tryptone soft agar and overlaid on hard agar and plaque forming units (PFU) were observed after incubated overnight at 30°C. Isolated plaques were pooled and concentrated using PEG (8000) precipitation method (30).

Susceptibility Analysis

Phage host range was examined using the spot test method (3). Soft agar (3-5 mL) with 100 µl of an overnight bacterial culture and equal volume of $CaCl_2$ (300 mM) were gently vortexed and spread on the surface of hard agar. Single drops of each phage lysate were spotted on the inoculated hard agar plates, and the plates were incubated overnight at 30°C.

Phage DNA isolation

Phage DNA was extracted as reported earlier (16). Briefly, 0.5% of SDS and proteinase K (0.05mg/mL) were added to 0.5ml of phage lysate and heated for 15-30 minutes in a 65°C water bath. A 1:1 phenol:chloroform extraction was performed 4 times followed by incubation with 0.3M Sodium acetate and 3 volumes of 100% cold ethanol at -20°C for 30 minutes. Then 70% ethanol wash was performed 3 times and then the DNA was air dried and resuspended in TE buffer.

Restriction analysis of phage DNA

The Phage DNA isolates were digested with restriction enzymes using a standard restriction digestion assay (32) as per the manufacturer's instructions (Fermentas). Of the five enzymes tested (Eco RI, Hind III, Nhe I, Kpn I and Alu I) two were selected (Eco RI, Hind III) based on the observation of clear multiple bands on agarose gels. The restriction digests were electrophoresed on 0.8% agarose gels in 1 X TAE buffer stained with ethidium bromide and visualized by UV photography. The phage genome sizes were determined by using semi-log plotting method where, a logarithmic scale is used to graph the results in semi-log paper. (11). Restriction digested samples run on the agarose gel along with the 1 kb ladder marker was used for semi-log plotting. The sum of the size of the bands present in each sample is the approximate size of the bacteriophage genome.

SDS-PAGE analysis of phage proteins

Phage structural proteins were analyzed by sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) as described by Laemmli (20). Samples were heated at 95°C for 15 min and subjected to electrophoresis with a 10% polyacrylamide gel at 150 V for 2 hrs. Proteins were visualized on gels stained with silver nitrate (7).

AFM analysis

The sample was prepared as described by Archer et al., (4). The virus particles were deposited for adsorption on preliminary cleaned, glass cover slips. After adsorption of the phage particles onto the surface, the substrates were allowed to dry at room temperature and then carefully rinsed with Milli-Q water. Imaging was performed using NTMDT-Atomic Force Microscopy.

RESULTS

A total of 15 fluorescent bacteria were isolated from both soil and sewage samples. Each soil sample was enriched with soil isolates as their isolation host. Plaques were observed only with phage lysates obtained from sewage. In total, 7 lytic phages were isolated from sewage enrichment process. They were selected based on their size and morphology of the plaques. The size and plaques produced by the isolated phages in all three months are listed in Table 1. The bacteriophage load on both inlet and outlet of sewage treatment plant was also assessed. Ninety percent of bacteriophage reduction was found in outlet sample (data not shown).

The susceptibility of the bacterial isolates to infection by 7 phages was examined. All the 7 phages isolated, individually lysed the sewage bacterial isolates Pfsw-13 and Pfsw-15 by the spot test method. Phage #5 showed lytic activity against several fluorescent isolates and also against *P. aeruginosa* MTCC 2474 standard strain. The susceptibility of the bacterial isolates to these phages is shown in Table 2.

Table 1. Morphology and size of the plaques

Phage isolates from sewage	Plaque size (mm dia)	Plaque morphology	PFU/ml
#1	2 mm	clear	5.6×10^7
#2	5 mm	turbid	3.6×10^7
#3	3 mm	turbid	2.3×10^7
#4	1 mm	clear	3.2×10^7
#5	2 mm	clear	5.9×10^7
#6	2 mm	clear	1.4×10^7
#7	1 mm	clear	3.2×10^7

Table 2. Bacterial susceptibility to *Pseudomonas* phages

HOST	SOURCE	PHAGE ISOLATES						
		#1	#2	#3	#4	#5	#6	#7
Pfsw-13	Sewage treatment plant	S	S	S	S	S	S	S
Pfsw-14	Sewage treatment plant	R	R	R	R	R	R	R
Pfsw-15	Sewage treatment plant	S	S	S	S	S	S	S
P.fluorescens	Reference strain	S	R	S	S	S	R	S
P.aeruginosa	Reference strain	R	S	R	R	S	S	R

(R- resistant, S- susceptible)

Genome fingerprinting analysis

The DNA isolated from all the phages were double stranded and were sensitive to EcoRI and Hind III. Figure 1 shows the restriction pattern of the phage DNA. The estimated genome sizes ranged from 26 to 82 Kb. The genome size of each phage isolates was found to be similar when tested by the two enzymes used. The genome fingerprint of the phage DNA shows the diversity among the phage isolates.

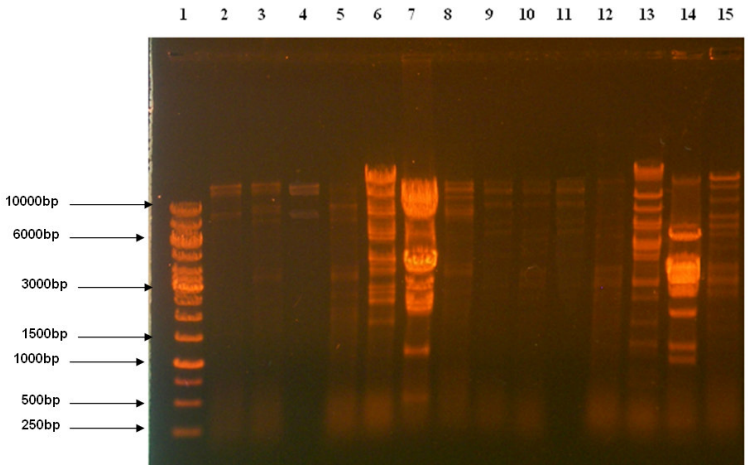

Figure 1. Restriction profile of the isolated bacteriophage DNA
Lane 1- 1Kb ladder
Lane 2- 8: EcoRI digestion of the phage DNA
Lane 9-15: HindIII digestion of phage DNA samples
(Phage #1 to #7 respectively)

Analysis of phage proteins

Phage lysates were examined using SDS-PAGE, stained with silver nitrate. Multiple bands were present in each of the lysates. At least 5 bands can be clearly distinguished in the gel ranging from approximately 97 kDa to 14 kDa (Figure 2). Phage #5 isolate has a distinct band at a size of approximately 35 kDa with high concentration.

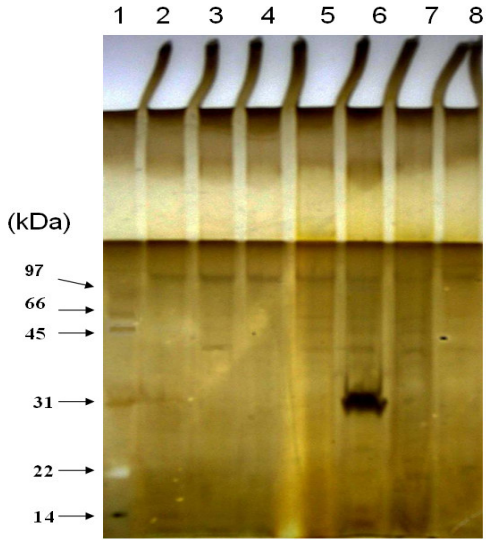

Figure 2. Protein profile of the bacteriophages on SDS PAGE, stained with silver nitrate
Lane 1: Standard protein marker
Lane 2-8: Whole cell protein of the bacteriophage isolates

AFM analysis

Fig 3 shows atomic force microscopy topological images of bacteriophage particles on aminosilanized glass. Phage size measurements were performed directly on micrographs at 1µm magnification. According to the head diameter, viral particles were classified into three size classes (>= 85 nm, >40nm and < 15nm). Bacteriophages could not be clearly differentiated according to their fine structures.

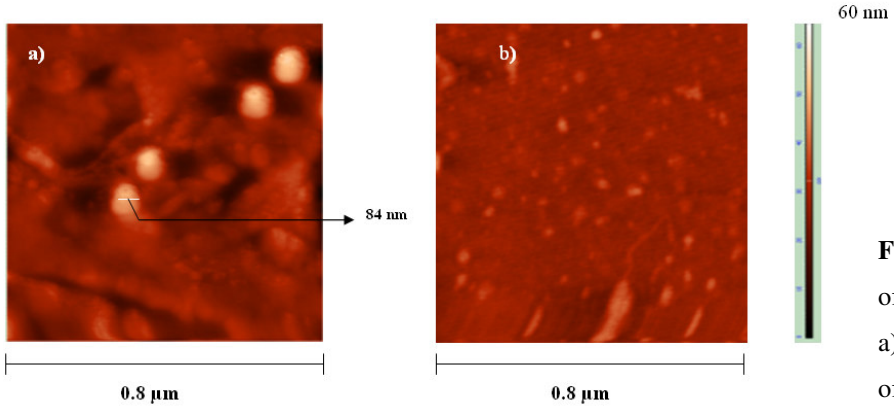

Figure 3. Atomic force microscopy images of bacteriophages
a) Morphology of phage #5, b) morphology of phage #1.

DISCUSSION

Seven phages were isolated from sewage enrichment process, whereas isolation of phages from soil samples was found to be unsuccessful. The results are in concordance with an earlier study wherein the phages of *Pseudomonas* were successfully isolated only from water samples, while in soil and feces no *Pseudomonas* specific phages were detected (22).Earlier studies attribute similar results to relatively low viable counts of phage capable of infecting specific bacteria (6), relatively low rates of phage diffusion within soil, particularly under drier conditions (18) and relatively high rates of free-phage inactivation within soil (27). There have been a number of reports of phage presence, in various environments (14,2). However, it is uncertain how those phages from sewage have impact on bacterial populations in soil environment. The susceptibility analysis of soil bacterial isolates in this study shows the soil bacterial population was resistant to these phage isolates. It is interesting to note that soil bacterial isolates obtained within the vicinity of sewage treatment plant showed resistance to the phages isolated from sewage. Each of the 7 phages was able to individually lyse the isolation host. None of the *P. fluorescens* bacteria isolated from the cultivated soils were susceptible to seven phages. In one study, phages of human enteric bacteria were also reported to be associated with plants displaying broad host range (29). Similarly, phage infecting phytopathogenic *Pseudomonas* have been isolated from sewage (21, 36) while *Agrobacterium*-infecting phage have been isolated from feces (25, 31). But from our study, it was found that the bacteriophage having broad host range activity isolated from sewage environment showed no lytic activity among cultivated soil isolates. However, further studies need to be carried out to understand the impact on natural bacterial population of cultivated soils. The phage #5 in our study was able to completely lyse the host bacterium *P. fluorescens* and *P. aeruginosa*. This indicated the broad range of host specificity of the bacteriophage. Broad host range phages play a key role in phage ecology and gene transfer in nature (14). The restriction pattern of the phage DNA shows the genomic diversity among the isolates. All the phages

isolated were double stranded and sensitive to EcoRI and HindIII (Figure 1). The restriction analysis indicated that the phages were genetically distinct. The phage #6 has a genome size of about 37 kb which is very close to the *P. aeruginosa* phage MP22 (12) and also phage #2 has approximately 58 kb genome size, similar to D3 phage which is a *P. aeruginosa* phage (19). The host range of these two phages also suggests that they are lytic against *P. aeruginosa* (Table 1). The phage #5 has a genome size of about 80 kb, which showed broad range host specificity and did not have match to any sequences of the phage genome databases. A major protein with the same molecular weight (above 97 kDa) was present in all the seven phages analyzed, which is consistent with the results obtained by Alonso et al., (2). It suggested that the molecular weights of the major proteins do not vary among phage lysates specific to the same bacterial strains. The morphological feature of the phage #5 was also different from other six phage isolates. The average size of the capsid for bacteriophage #5 was approximately 84nm. This phage has morphological characteristics somewhat similar to T4 bacteriophage (4). However, the fine structure like tail length was not observed. The ecological importance of this phage must be further studied. Though only representative part of the *Pseudomonas* phages was investigated, a great variability has been observed.

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